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Advanced glycation end products impair protein turnover in LLC-PK$_1$: Amelioration by trypsin

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Background. Advanced glycation end products (AGEs) are assumed to play a key role in the pathogenesis of diabetic nephropathy (DN) and other diabetic complications. While AGEs have been shown to exert marked effects on mesangial and endothelial cells as well as on monocytes/macrophages, little is known about their effects on tubule cells. Therefore, we addressed the questions of (*1*) whether AGE-bovine serum albumin (AGE-BSA) impairs the protein metabolism in the tubule cells, and if so, (*2*) whether the AGE-induced effects are mediated via a protease sensitive mechanism.

Methods. Arrested LLC-PK$_1$ cells were exposed to a medium containing the vehicle (control, serum free), AGE-BSA (38 μmol/L), or BSA (38 μmol/L) in the presence or absence of trypsin (2.5 μg/mL) for 24 hours. We evaluated cell number, cell size, and cell protein content, as well as protein synthesis and protein degradation.

Results. After an incubation period of 24 hours, AGE-BSA decreased the cell number to 84.5 ± 5.5% of control and 82.5 ± 5.6% of BSA-treated cells ($P < 0.05$). [^{3}H]-thymidine incorporation declined to 66% of control ($P < 0.05$), while BSA was without any effect. The same AGE-BSA dose reduced protein degradation ($P < 0.05$) and stimulated total protein synthesis slightly, as determined by L-[^{14}C]Phe incorporation into acidic-insoluble proteins. These effects resulted in a rise in cell protein content (AGE-BSA vs. control, 21.9 ± 6.7%; AGE-BSA vs. BSA, 11.1 ± 6.0%, $P < 0.05$) and cell volume (AGE-BSA vs. control 9.4 ± 3.2%, AGE-BSA vs. BSA 18.4 ± 3.7%, $P < 0.05$). Coincubation with AGE-BSA and trypsin was associated with an amelioration of all investigated parameters concerning cell number, cell proliferation, raised cell protein content, decreased protein degradation, and enhanced protein synthesis.

Conclusion. These data indicate that AGE-BSA impairs cell proliferation and protein turnover in LLC-PK$_1$ cells with a consequent rise in cell protein. Since these alterations were abrogated by coincubation with trypsin, an interference of this serine protease with the AGE-binding proteins on cell surfaces is assumed.

Key words: diabetic nephropathy, tubule cells, protein metabolism, cell proliferation, serine protease.

Diabetic nephropathy (DN) is characterized by renal hypertrophy, thickening of basement membranes, and accumulation of extracellular matrix (ECM) in the mesangium and tubulointerstitium [1, 2], and results both from enhanced production of proteins as well as from impaired protein degradation (PD) [3–6]. In the pathogenesis of DN, in addition to hyperglycemia, enhanced formation of transforming growth factor-β$_1$ (TGF-β$_1$) and activation of the renin-angiotensin system (RAS), early and advanced glycation end products (AGEs) are assumed to play a key role [7–11]. AGEs accumulate in the glomeruli and tubulointerstitium in relationship to severity of renal disease [12, 13], and administration of AGE-BSA to normal rats and mice led to lesions similar to those of DN [14, 15]. In in vitro studies, AGEs elicited a multitude of toxic effects such as enhanced formation of oxygen radicals, release of cytokines (in particular TGF-β$_1$), expression of adhesion molecules, and enhanced vascular permeability [16–18]. These responses most likely are mediated by AGE receptors, among which the receptor for AGEs (RAGE) is the best studied member [19–21]. Binding of AGEs to RAGE results in the activation of the transcription factor nuclear factor-κB as well as activator protein-1 (AP-1) complex [22–24].

In contrast to the numerous studies performed in mesangial and endothelial cells, there are only minimal data concerning AGE effects on tubular cells, the main target of renal hypertrophy in diabetes. Therefore, in the current study, the influence of AGE-BSA on proliferation and protein turnover of tubule cells was investigated. Furthermore, the effect of coadministered trypsin was examined, since AGE-binding proteins at surfaces of endothelial cells were shown to be sensitive to this serine protease [25, 26]. Thereby, an inactivation of the AGE receptor may protect the cells from maladaptive responses.

Results of the present study showed that exposure of LLC-PK$_1$ cells to AGE-BSA reduced cell number and

[^{3}H]-thymidine incorporation. Cell protein content and cell volume were increased because of impaired PD and slightly enhanced protein synthesis (PS). Coadministration of trypsin abrogated these alterations, most likely due to an interference of this serine protease with binding proteins of AGE-BSA at cell surfaces.

METHODS

Generation and characterization of AGE-modified albumin

Bovine serum albumin (1 mmol/L; BSA fraction V, Sigma, Germany) was glycated by incubation with 1 mol/L glucose in 50 mmol/L potassium phosphate, pH 7.3, 1 mmol/L ethylenediaminetetraacetic acid (EDTA) under sterile conditions at 50°C for 40 days according to a modified protocol of Schmidt et al [25].

Cell culture and experimental treatment

LLC-PK$_1$ cells, a porcine cell line that exhibits properties of proximal tubules, were grown at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS; GIBCO, Grand Island, NY, USA), 25 mmol/L HEPES, 100 U/mL penicillin, and 100 ng/mL streptomycin. Cells were plated separately on 100 mm dishes (isolation of RNA), 6-well plates (cell proliferation assay), or 12-well plates (protein turnover measurements). Subconfluent cells were synchronized by serum-free medium (SFM) for 24 hours, followed by treatment with vehicle (control), BSA or AGE-BSA (19 to 76 μmol/L) related to BSA content with or without trypsin (0.625 to 5 μg/mL final concentration) for 24 hours. Cytotoxicity was evaluated both by trypan blue exclusion and release of lactate dehydrogenase (LDH). Cell number and cell volume was analyzed using the Casy-1 System (Schaerfe, Reutlingen, Germany).

[^{3}H]-thymidine incorporation assay

The subconfluent monolayer was quiescented by incubation with serum-free DMEM for 24 hours and then incubated for another 24 hours in SFM in the absence (control) or the presence of AGE-BSA or nonglycated BSA with or without trypsin at various concentrations. During the last four hours, cells were labeled with 0.5 mCi/well of [^{3}H]-thymidine (50 μCi-mmol; Amersham, Frankfurt/M, Germany). The cells were rapidly washed three times with ice-cold phosphate-buffered saline (PBS) and solubilized in 2% sodium dodecyl sulfate (SDS), followed by precipitation with 1 mL of 20% trichloroacetic acid. The precipitates were collected with a cell harvester onto a glass microfiber filter (Schleicher and Schuell, Dassel, Germany) and washed sequentially with 10 and 5% trichloroacetic acid (TCA) and finally with ethanol. Incorporated [^{3}H]-thymidine was measured by a liquid scintillation counter.

Cellular protein content

Total protein concentration in cell lysate was measured using bicinochoninic acid (BCA protein assay; Pierce, Rockford, IL, USA) [27].

Protein synthesis

Protein synthesis was assessed by determining the incorporation of L-[^{14}C] phenylalanine (Phe; Amersham) into acid-insoluble proteins [28]. Subconfluent cells were preincubated for two hours in media containing 0.6 mmol/L unlabeled Phe, AGE-BSA, unmodified BSA, or vehicle (as control) before changing to a medium containing L-[^{14}C] Phe (0.5 μCi/well) plus 0.6 mmol/L unlabeled Phe for 24 hours. The excess of unlabeled Phe (0.6 mmol/L) was used to ensure the equilibrium of intracellular- and extracellular-specific radioactivity. At the end of the four-hour labeling period, the cells were rapidly rinsed three times with ice-cold PBS. The cells were then solubilized in 2% SDS and precipitated. The radioactivity in the cells was determined in a Tri-Carb 4000 liquid scintillation counter (Packard Instruments, Downers Grove, CA, USA). The cell PS rate was expressed as dpm per 10^5 cells.

Protein degradation

To measure PD rate, the quiescent subconfluent cells were labeled by 0.5 μCi/well of L-[^{14}C]Phe for 24 hours [28]. To minimize the reincorporation of L-[^{14}C]Phe, the experimental media contained an excess (2 mmol/L) of unlabeled Phe. After two hours, the cells were washed with ice-cold PBS to remove L-[^{14}C]Phe released from the degradation of short-lived proteins; 4 mL of fresh experimental medium (control) containing BSA or AGE-BSA were added with and without trypsin. At the indicated time points, 0.5 mL aliquots of the media were collected and precipitated by adding ice-cold TCA (10% final concentration). The samples were centrifuged for five minutes, and the TCA-soluble supernatants were stored at −20°C. At the end of an experiment, the cells were washed three times with ice-cold PBS. Radioactivity in cells and media was measured as mentioned previously in this article. The percentage of radioactivity released versus the radioactivity remaining in the cells at the indicated time intervals was calculated as the PD rate and expressed as cpm/10^5 cells.

Statistical analysis

The mean for the replicates of each experiment was determined. Results are presented as mean ± SD, with N indicating the number of experiments. A comparison between the two groups was made using paired and unpaired t-test. Group differences (>3) were evaluated em-

Table 1. Effect of different treatments on cell number, protein content and cell size after 24 hours of incubation

	Cell number $\times 10^6$	Protein content *ng/cell*	Cell volume *fL/10³*
Control	3.95 ± 0.12	0.730 ± 0.07	2827.79 ± 101.6
AGE-BSA	3.34 ± 0.22[a]	0.894 ± 0.06[a]	3092.06 ± 101.6[a]
BSA	4.05 ± 0.35	0.806 ± 0.01	2611.85 ± 96.7
AGE-BSA + Tryp	4.01 ± 0.30	0.772 ± 0.05	2632.97 ± 97.56
BSA + Tryp	4.23 ± 0.24	0.810 ± 0.08	2865.89 ± 110.1

LLC-PK$_1$ cells were grown in vehicle, advanced glycation end products-bovine serum albumin (AGE-BSA) (38 μmol/L), and BSA (38 μmol/L) with or without trypsin (Tryp; 2.5 μg/mL) for 24 hours. Cell monolayers were harvested by 0.25% trypsin/0.02% EDTA to obtain a single cell suspension, and subjected to the measurements of cell volume and cell number. Values are expressed as the mean ± SD from three independent experiments ($N = 9$).

[a] $P < 0.05$ vs control, BSA, AGE-BSA + Tryp

ploying analysis of variance. Statistical significance was defined at $P < 0.05$.

RESULTS

Effect of AGE-BSA on cell proliferation

With AGE-BSA (38 μmol/L), treatment of LLC-PK$_1$ cells, cell number was reduced within 24 hours to 84.5 ± 5.6% in comparison to control ($P < 0.05$, $N = 9$) and to 82.5 ± 5.5% in comparison to BSA-treated cells ($P < 0.05$, $N = 9$; Table 1). This reduction was not due to cell death, since both exclusion of trypan blue and release of LDH were not significantly changed in the different groups. [^{3}H]-thymidine incorporation decreased within 24 hours from 2678 ± 170 control to 1857 ± 178 cmp/well ($P < 0.05$). Coadministration of trypsin enhanced the incorporation to 2321 ± 214 cmp/well ($P < 0.05$).

Effect of AGE-BSA on cell volume and cellular protein content

In the cells grown for 24 hours in AGE-BSA (38 μmol/L), volume increased by 9.4 ± 3.2% ($P < 0.05$) as compared with untreated cells and by 18.4 ± 3.7% ($P < 0.05$) as compared with BSA-treated cells (Table 1). Coincubation of the cells with trypsin (2.5 μg/mL) reversed this AGE-BSA effect.

Advanced glycation end product-BSA (38 μmol/L) for 24 hours increased cellular protein content by 21.9 ± 6.7% as compared with control (0.89 ± 0.06 vs. 0.73 ± 0.07 ng/cell, $P < 0.05$) and by 11.1 ± 6.0% as compared with BSA-treated cells (0.89 ± 0.06 vs. 0.80 ± 0.01 ng/cell, $P < 0.05$). Protein content of cells grown in BSA was not significantly altered as compared with untreated cells. Coincubation of AGE-BSA with trypsin (2.5 μg/mL) abolished the rise of cell protein content.

Effect of AGE-BSA on PS

To exclude binding of AGE-BSA to cells as the main reason for the measured increase in cellular protein content, effects of AGE-BSA on cellular PS and degradation were analyzed in addition. [^{14}C]-Phe incorporation into TCA-precipitated cell protein was slightly increased in cells exposed to AGE-BSA (38 μmol/L) in comparison to cells exposed to BSA (38 μmol/L). The labeling incorporation rate after 12 and 24 hours was 117.6 ± 2.2% ($P < 0.05$) and 109.9 ± 10.5% (P = NS), respectively, when compared with the same concentration of BSA. Coincubation with trypsin (2.5 μg/mL) prevented the rise in PS.

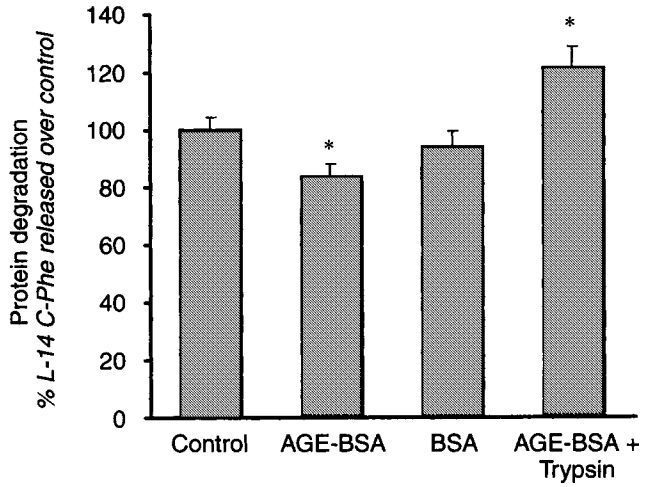

Fig. 1. Effect of advanced glycation end product-bovine serum albumin (AGE-BSA) and BSA on intracellular protein degradation in LLC-PK$_1$ cells. Exposure to AGE-BSA (38 μmol/L) decreased protein degradation to 83% of controls after 24 hours. Coincubation of AGE-BSA with trypsin antagonized the AGE effect. Data are given as means ± SD of eight determinations from two separate experiments. *$P < 0.05$ vs. BSA-treated, AGE + trypsin-treated cells.

Effect of AGE-BSA on protein degradation

After cells exposure to AGE-BSA (38 μmol/L) for 24 hours, the release of incorporated [^{14}C]-Phe was reduced by 17 ± 7% as compared with the control ($P < 0.05$, $N = 6$) and by 10 ± 6% as compared with BSA-treated cells (NS, $N = 6$; Fig. 1). Coincubation of the cells with trypsin (2.5 μg/mL) increased the rate of PD by 37 ± 5.7% as compared with AGE-BSA treatment alone ($P < 0.05$, $N = 6$).

DISCUSSION

In the current investigation, AGE-BSA and not BSA inhibited the proliferation of LLC-PK_1 cells, as demonstrated by a decrease in cell number and [^{3}H]-thymidine incorporation without any effects on cell viability and LDH release. These findings are consistent with studies in mesangial [29, 30], smooth muscle [31], and endothelial cells [32], as well as retinal capillary pericytes [32].

The antiproliferative action of AGE-BSA in LLC-PK_1 cells was associated with a rise in cell volume and cell protein content. PS (as demonstrated by incorporation of L-^{14}C Phe) was slightly increased, while PD was significantly reduced. The latter finding might be explained, at least in part, by impaired proteolytic activities. Thus, in our earlier study, incubation of LLC-PK_1 cells with AGE-BSA caused a marked decline of cathepsin B + L activities caused by lowered enzyme expression [33].

These alterations are most probably caused by an AGE-BSA–induced overexpression of TGF-β_1, which was shown to enhance PS on the one hand and to impair PD on the other [34, 35]. Indeed, in our own preliminary studies, TGF-β_1 overexpression was observed in porcine LLC-PK_1 cells after AGE-BSA incubation (abstract; Xiang et al, *J Am Soc Nephrol* 9:629, 1998), corresponding with data in mesangial cells [16]. Additionally, in vivo administration of AGE-BSA in normal rats and mice led to an overexpression of TGF-β_1 in the glomeruli [15].

To prevent the intracellular/extracellular effects of AGEs, various experimental and clinical approaches have been employed. Administration of aminoguanidine, a blocker of AGE formation, attenuated the renal pathology of streptozotocin (STZ)-diabetic rats [36]. Additionally, antioxidants (taurine, vitamin E, and alpha-lipoic acid) protected mesangial and/or endothelial cells from AGEs and/or glucose induced injury, probably by decreased activation of nuclear factor-κB and/or TGF-β_1, respectively [24, 37]. Recently, administration of the soluble receptor of AGEs was reported to ameliorate diabetic atherosclerosis [38].

In our study, we investigated the question of whether trypsin modulates the effect of AGE-BSA in LLC-PK_1 cells, since this protease inhibits the protein binding of AGEs at the surface of endothelial cells [25, 26]. Our data show that coincubation of AGE-BSA with trypsin ameliorates the antiproliferative effects, the raised cell protein content, as well as the increased PS, and impairs PD. In preliminary investigations, we could also observe a decrease of intracellular AGE accumulation in LLC-PK_1 cells, as determined by an immunohistochemical staining method of imidazolone, which could explain the beneficial effects of trypsin on the investigated parameters (data not shown).

The question arises, how could AGEs exert their toxic effects in tubular cells in DN? Contact with the basolateral membrane is determined by the blood concentration of AGEs and at the apical site by the glomerular filtration of the low molecular weight AGE peptides. Thus, after injections of AGE containing peptides in normal rats, a reabsorption of these compounds by proximal tubules has been demonstrated [39]. Moreover, in the presence of glomerular proteinuria, besides AGE peptides the higher molecular weight AGE proteins also are translocated into the proximal tubular fluid. Here, they may interact with cell receptors at the apical site with consequent cell activation. Recently, binding sites specific for AGEs were demonstrated within the proximal tubule cells of the rat kidney [40].

In conclusion, our data show that AGE-BSA exerts marked effects on LLC-PK_1 cells, such as decreased proliferation, rise in cell volume and cell protein content, impaired PD, and slightly enhanced PS. These alterations are substantially inhibited by coadministration of trypsin. It is assumed that this serine protease hinders the interaction of AGEs with cell binding proteins, thereby preventing the release of cytokines.

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